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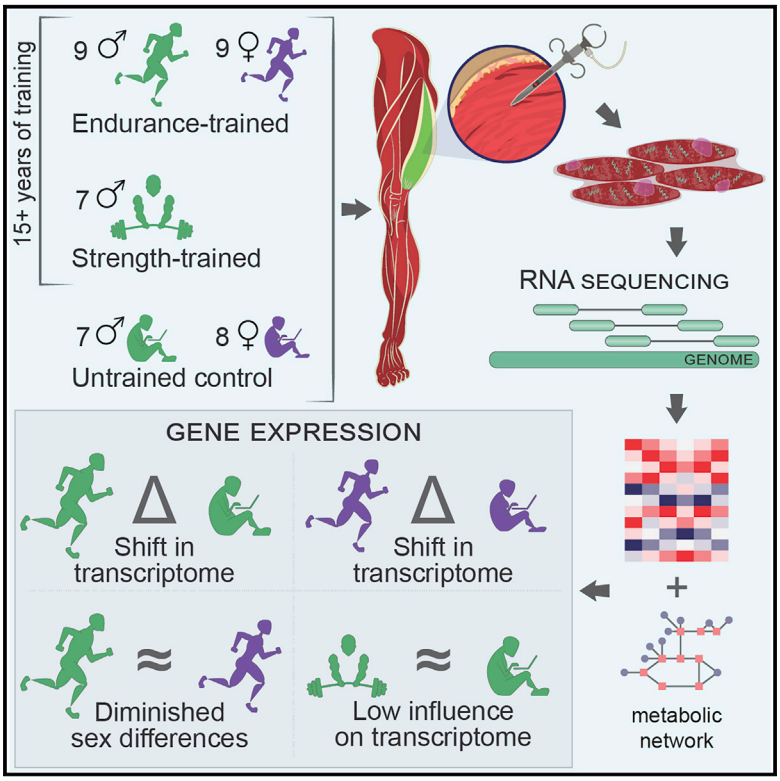
Skeletal Muscle Transcriptomic Comparison between Long-Term Trained and Untrained Men and Women

Graphical Abstract



Highlights

- Endurance training shifts the transcriptome significantly compared with controls
- Sex differences in the muscle transcriptome are diminished with endurance training
- Strength training appears to not have a large effect on the resting transcriptome
- A comparative analysis reveals genes that could attenuate metabolic diseases

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In Brief

Exercise training performed over a lifetime is known to result in numerous health benefits and can prevent diseases, such as type 2 diabetes, heart disease, and even cancer. To understand how long-term exercise can lead to such benefits, Chapman et al. compared gene expression levels in long-term-trained men and women.

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Skeletal Muscle Transcriptomic Comparison between Long-Term Trained and Untrained Men and Women

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SUMMARY

To better understand the health benefits of lifelong exercise in humans, we conduct global skeletal muscle transcriptomic analyses of long-term endurance- (9 men, 9 women) and strength-trained (7 men) humans compared with age-matched untrained controls (7 men, 8 women). Transcriptomic analysis, Gene Ontology, and genome-scale metabolic modeling demonstrate changes in pathways related to the prevention of metabolic diseases, particularly with endurance training. Our data also show prominent sex differences between controls and that these differences are reduced with endurance training. Additionally, we compare our data with studies examining muscle gene expression before and after a months-long training period in individuals with metabolic diseases. This analysis reveals that training shifts gene expression in individuals with impaired metabolism to become more similar to our endurance-trained group. Overall, our data provide an extensive examination of the accumulated transcriptional changes that occur with decades-long training and identify important "exercise-responsive" genes that could attenuate metabolic disease.

INTRODUCTION

Physical exercise specifically alters skeletal muscle in an exercise-modality-dependent manner. Mitochondrial content, capillary density, and various metabolic substrate transporters are all increased with endurance training. With regards to strength training, muscle hypertrophy is the most prominent change. Tissue alterations common to both exercise modalities are improved insulin sensitivity, increased muscle glycogen, and a shift in skeletal muscle fiber type composition (Lieber, 2010). These adaptations can largely be attributed to changes in gene activity and post-translational protein modifications in response to repeated exercise bouts (Chapman and Sundberg, 2019). In addition to these changes in skeletal muscle, regular physical activity has numerous specific health benefits. These include improved well-being, increased quality of life, lengthened lifespan, enhanced cognitive function, and the prevention and treatment of various diseases, including cardiovascular disease, diabetes, sarcopenia, osteoporosis, and cancer (Neufer et al., 2015; Mijwel et al., 2018). Although the benefits of physical activity are well documented, the mechanisms behind how physical activity promotes health and prevents disease are less understood. Given this lack of mechanistic knowledge, grant support for research in the area of physical activity has recently been prioritized (Neufer et al., 2015). Understanding the mechanisms of action behind the massively beneficial effects of physical activity can bring us closer to developing therapies for individuals for whom regular physical activity is not possible, such as in patients with cachexia, paralysis, severe joint pain, or in the morbidly obese.

Large-scale transcriptomic data have been very useful in describing and identifying the molecular factors involved in muscle adaptation to physical activity, particularly with endurance training (Mahoney et al., 2005; Timmons et al., 2005; Stepto et al., 2009; Keller et al., 2011; Lindholm et al., 2014a). Such studies are important investigations into the transcriptomic events involved in muscle plasticity. However, those studies have primarily examined either acute responses to training or short-term training periods of less than 1 year, and investigations of chronically (>15 year) trained athletes are scarce. Although understanding acute adaptation to exercise is interesting and useful, it is important to emphasize that regular exercise performed over decades is what profoundly promotes health and prevents disease. Thus, the objective of this study was to investigate skeletal

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Table 1. Research Subject Characteristics.

Group	Sex	Sample Size	Age (Years)	Weight (kg)	Height (cm)
Control	male	7	41.7 ± 4.4	77.4 ± 11.9	180.1 ± 10.1
Control	female	8	43.5 ± 5.2	65.9 ± 5.0	170.9 ± 7.8
Endurance	male	9	40.9 ± 2.8	75.6 ± 5.7	181.4 ± 7.3
Endurance	female	9	41.8 ± 5.9	57.1 ± 4.4	169.8 ± 4.3
Strength	male	7	42.1 ± 5.8	91.4 ± 11.5	181.9 ± 7.7

Data are presented as mean ± SD. See also Table S1 for a complete summary of self-reported training history of all exercise-trained subjects.

muscle transcriptomics in long-term endurance-trained and long-term strength-trained humans compared with healthy controls to understand how skeletal muscle adapts to lifelong training.

Transcriptomics is a valuable resource, but proper analysis and interpretation of the resulting data are critical to obtain relevant mechanistic information. To this end, genome-scale metabolic models (GEMs) have been developed to provide context for large-scale –omics data (Mardinoglu et al., 2013; Väremo et al., 2013b; Väremo et al., 2015; Bordbar et al., 2014; Mardinoglu et al., 2018). GEMs are a list of metabolic equations incorporated into a network that links common metabolites. Each of these metabolic equations are placed into the appropriate cellular compartment and related to genes coding for each particular protein in the metabolic reaction. Incorporating transcriptomic data with proteomic data, GEMs provide insights into disease mechanisms, novel biomarkers, and drug discovery and have the potential to reshape our understanding of how skeletal muscle adapts to physical activity (Väremo et al., 2013b, 2015). This systems biology approach has recently been used to develop a GEM for human myocytes by merging transcriptomic data from *in vitro* muscle cells with existing proteomic data in the Human Protein Atlas (Väremo et al., 2015). That study reconstructed skeletal myocyte metabolism and cross-referenced the GEM with existing data from patients with type 2 diabetes (T2D) to identify a "metabolic signature" of how skeletal muscle metabolism is altered in T2D. Despite the high-impact nature of this study, the transcriptomic data originated from cultured myocytes, which does not fully represent muscle metabolism in the more relevant *in vivo* context. The creation of *in vivo* skeletal muscle GEM data holds enormous promise for obtaining a global and unbiased insight into skeletal muscle function, effects of physical activity on metabolism, and sex-specific skeletal muscle metabolism in trained and non-trained individuals. Such data could provide the foundation for future interventions and therapies aimed at reshaping skeletal muscle metabolism in individuals afflicted with metabolic disorders such as T2D and obesity.

Much of exercise physiology knowledge is primarily based on studies of male subjects. Thus, it is unclear how molecular and biochemical factors in skeletal muscle of female subjects are altered in response to acute exercise or long-term exercise training. This is highlighted by a series of experiments in male and female elite cyclists (Rowlands et al., 2008; Rowlands and Wadsworth, 2011). Furthermore, RNA sequencing (RNA-seq) has been used to sensitively detect over 10,000 genes expressed in human muscle and showed that approximately 3,000 genes are differentially expressed between women and men at baseline (Lindholm et al., 2014b). These studies suggest that molecular adaptations to exercise may be different in women versus men; thus, it is critical that exercise physiology studies include both sexes.

Understanding the regulation of human adaptation to physical activity has the potential to broaden fundamental biological knowledge. Furthermore, determining the cellular and molecular mechanisms behind the benefits of physical activity is critical for discovering novel strategies and targets for disease prevention and treatment. Thus, the purpose of this research is to mechanistically determine how long-term strength or endurance training influence skeletal muscle gene expression and metabolism in both men and women. Furthermore, we aimed to predict certain genes and pathways that appear to be important in the amelioration of metabolic diseases, such as diabetes.

RESULTS

Study Overview

Forty healthy male and female volunteers participated in this study (Table 1). All participants recruited were between the ages of 34–53, with an approximate average age of each group of 42 years old (Table 1). Endurance-trained men and women included in this study were primarily involved in running, cycling, or a combination of the two for at least 15 years (Table S1). Strength-trained men included in this study reported diverse strength training regimens for at least 15 years, including activities that would significantly activate the quadriceps, such as squatting and deadlifting (Table S1). Additionally, Table S1 contains a complete breakdown of each subject's self-reported training history (years) and average frequency of training during the past 15 years (reported in both days/week and h/week).

By design, physiological test results demonstrate a clear separation in the endurance capacity (VO_2 peak) of the male and female endurance-trained subjects (MEs and FEs, respectively) compared with the other groups (Figure 1A; Figure S1A). Furthermore, maximal quadriceps torque production in the male strength-trained subjects (MSs) was significantly higher and showed no overlap with any other group (Figure 1B; Figure S1B).

To verify the well-trained phenotypes depicted in Figure 1, basic muscle physiological parameters were assessed in the biopsies. First, citrate synthase (CS) activity was determined to estimate mitochondrial content (i.e., oxidative capacity). In agreement with the endurance capacity of our research subjects, CS activity was significantly higher in both the ME and FE groups than in the other groups (Figure 1C). Muscle fiber type is defined by the myosin heavy chain (MyHC) isoform present—slow-twitch muscle fibers contain MyHC I, fast-oxidative fibers contain MyHC IIa, and fast-glycolytic fibers are made up of MyHC IIx (Lieber, 2010). As expected, MEs and FEs both showed a significantly higher proportion of MyHC I than controls (Figures 1D and 1E). Additionally, MSs had a significantly higher proportion of MyHC IIa than MEs, which is indicative of a "strength-trained phenotype." Furthermore, both male and female control subjects (MCs and FCs, respectively) possessed a higher proportion of MyHC IIx than all other groups. The myofibrillar cross-sectional area (CSA) of laminin-stained transverse skeletal

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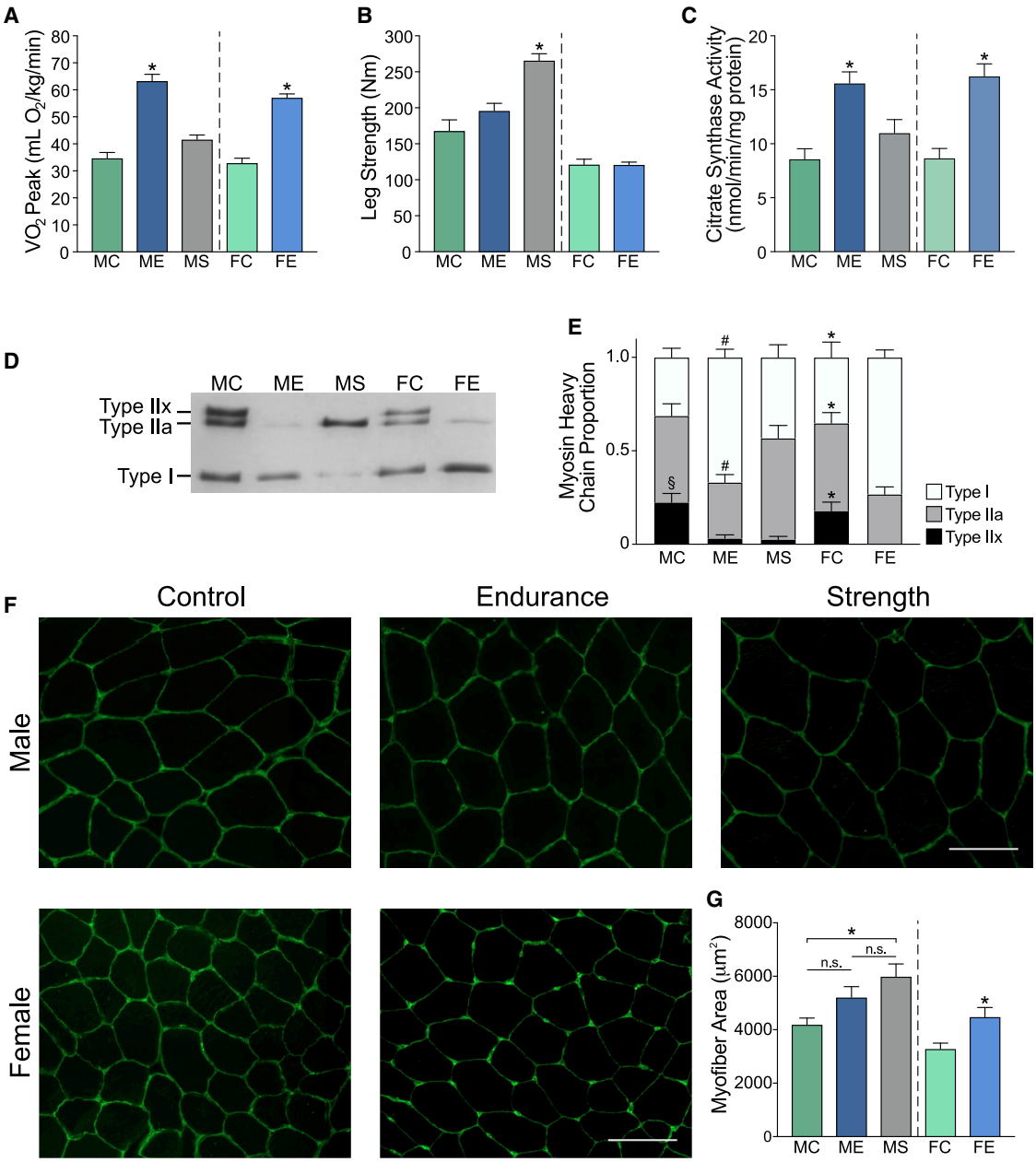

Figure 1. Physiological and Skeletal Muscle Biopsy Analyses Demonstrate a Clear Separation between the Endurance-Trained, Strength-Trained, and Control Subjects

(A) VO_2 peak was significantly elevated in male endurance-trained subjects (MEs, n = 9) compared with both male control (MCs, n = 7) and male strength-trained subjects (MSs, n = 7). Additionally, female endurance-trained subjects (FEs, n = 9) demonstrated a significantly higher VO_2 peak than female control subjects (FCs, n = 8).

(B) Isokinetic knee torque was significantly higher in MSs than in MEs and MCs. Isokinetic knee torque was not different between FEs and FCs.

(C) Citrate synthase (CS) activity is significantly increased in people with a history of endurance training. In male subjects, CS activity in endurance-trained individuals was higher than both MSs and MCs. FEs also demonstrated a significantly higher CS activity than FCs. These data strongly mirror the VO_2 peak data presented in (A).

(D) Myosin heavy chain isoforms differed based on previous training history. Myosin type I, IIa, and IIx isoforms are visualized by silver stain from each experimental group. Skeletal muscle from both male and female subjects without a history of exercise training (MCs and FCs) possess myosin type IIx. Endurance-trained subjects (MEs and FEs) possess a higher proportion of the slow (type I) myosin isoform. Strength-trained individuals have an increased proportion of the fast (type IIa) myosin isoform.

(E) Quantification of myosin heavy chain gels revealed that MCs had a significantly elevated amount of type IIx myosin than MEs and MSs. Additionally, MEs had a significantly higher proportion of type I myosin than MCs and MSs. Furthermore, the proportion of type IIa myosin was significantly lower in MEs than MCs and

(legend continued on next page)

muscle sections was assessed to determine the level of muscle hypertrophy (Figure 1F). In male subjects, myofibrillar CSA closely mirrored quadriceps torque data measured during subject screening (Figure 1G). Myofibrillar CSA in FEs was significantly higher than that in FCs (Figure 1G). The data presented thus far have verified that our physiological data presented in Figures 1A and 1B are concordant with the parameters measured in the skeletal muscle biopsies in Figures 1C–1G.

The Skeletal Muscle Transcriptome Primarily Differentiates Endurance Athletes from Sedentary Controls

With the robust phenotypic separation established between our different groups, we moved forward with further analyses of the transcriptional changes that accompany long-term exercise training. A summary of all of the differentially regulated genes found from the RNA-seq data is presented as an UpSet plot (Lex et al., 2014) and as a supplemental table (Figure 2A; Table S2). Samples were sequenced to an average depth of 41 million reads, with $\geq$96% of the data corresponding to protein-coding regions. Principal-component analysis (PCA) revealed a tight clustering of MEs as well as FEs (Figure 2B). Control subjects clustered together along with MSs (Figure 2B).

A total of 1,711 genes in FEs and 1,097 genes in MEs were found to be differentially regulated compared with the corresponding controls (Figure 2A; Table S2). Upon examination of Gene Ontology (GO), several similar pathways were significantly enhanced with endurance training in both men and women (Figures 3A and 3B; Table S3). Specifically, processes associated with cellular respiration and tricarboxylic acid (TCA) metabolism were significantly enhanced in long-term endurance-trained individuals of both sexes (Figures 3A and 3B; Table S3). In addition to differences in cellular respiration, FEs demonstrated an upregulation of genes related to protein turnover that was not observed in the MEs (Figure 3B). Many GO pathways were also found to be inhibited in long-term endurance-trained individuals (Figure S2). A large portion of pathways that were inhibited in both MEs and FEs were associated with the regulation of gene expression.

Significant Sex Differences Present between MCs and FCs

Upon examining sex differences at baseline, we found 452 differentially regulated genes when comparing MCs with FCs (Figure 2A). GO pathways related to protein catabolism were significantly elevated in MCs versus FCs (Figure 3C). Specifically, pathways related to protein ubiquitination, protein catabolic process, ribosome biogenesis, regulation of proteasomal protein catabolic process, and peptide metabolic process were all significantly enhanced in MCs versus FCs (Figure 3C; Table S3). Pathways related to wound healing and extracellular structure were highly enhanced in FCs compared with MCs (Figure S3A; Table S3). Additionally, although males generally exhibited a higher expression in pathways associated with peptide metabolism, female subjects showed an elevated expression of lipid metabolism (Table S3).

Sex Differences in the Skeletal Muscle Transcriptome Are Diminished in Endurance-Trained Athletes

Compared with the gene expression differences observed between MCs and FCs, many fewer genes were differentially regulated between MEs and FEs (Figure 2, 135 genes in total). GO pathway analysis showed that MEs had a higher oxidative metabolic signature, as displayed by an increased expression of mitochondrial-related and oxidative metabolic pathways (Figure 3D; Table S3). Peptide metabolism was also found to be elevated in male compared with female athletes but to a lesser extent than the sex differences found in controls (Figures 3C and 3D; Table S3).

Long-Term Strength Training Does Not Have a Large Influence on the Resting Skeletal Muscle Transcriptome

The fewest differentially expressed genes were found between MS and MC groups (Figure 2, 26 genes in total). Despite the small number of differentially regulated genes, GO pathway analysis revealed an upregulation in pathways primarily related to cellular respiration (Figure S4A; Table S3). Additionally, long-term resistance-trained men displayed a downregulation in pathways associated with the negative regulation of cell proliferation (Figure S4B; Table S3).

Genome-Scale Metabolic Modeling Confirms Activation of Metabolism with Endurance Training

We integrated transcriptomics data with genome-scale metabolic modeling and found that the activity of most of the metabolic pathways were activated in long-term trained endurance athletes. We observed that the expression of numerous components in the TCA cycle were elevated in both MEs and FEs compared with the corresponding controls (Figures 4A and 4B). We also found that catabolism of branched chain amino acids, including valine, leucine, and isoleucine, as well as the oxidation of fatty acids was significantly elevated in both endurance groups compared with the controls. Reporter metabolite analysis also showed that there were significant changes around central metabolites, including acetyl coenzyme A (Figures 4A and 4B). Based on the differential gene expression analysis, we found that the changes in the TCA cycle pathway were more pronounced in male athletes. Additionally, we observed that the fatty acid biosynthesis process was significantly enhanced in male athletes only.

MSs. When comparing FEs with FCs, there was a significant difference in all three fiber types. §, significantly different from both MEs and MSs; #, significantly different from MCs and MSs; *, significantly different from FEs.
(F) Transverse skeletal muscle tissue sections stained with laminin demonstrate that the cross-sectional area of myofibers is altered with long-term training.
(G) The myofibrillar cross-sectional area was significantly higher in MSs than in MCs. MEs had an intermediate muscle fiber area that was not significantly different from MCs nor MSs. FEs had a significantly higher muscle fiber area than FCs.
Scale bars, 100 μm. All data are presented as mean ± SEM and with significance, as indicated with *, #, or §, set to $p < 0.05$.
See also Figure S1 for a scatterplot of VO_2 peak and peak leg torque data for all subjects.

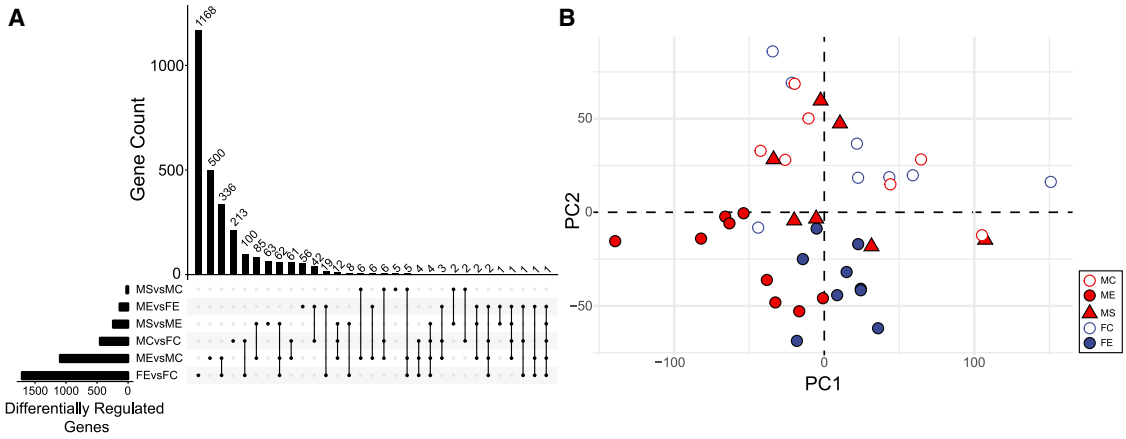

Figure 2. RNA Sequencing Data Show Large Baseline Differences between Endurance-Trained Subjects and Controls, as Well as Sex Differences

(A) UpSet plot representing the number of genes that are differentially regulated by exercise (MSs versus MCs, MSs versus MEs, MEs versus MCs, and FEs versus FCs) and by sex (MCs versus FCs and MEs versus FEs) (Lex et al., 2014). The horizontal bars represent the total number of genes differentially regulated in the listed comparison. The vertical bars represent the number of genes uniquely differentially regulated in the highlighted comparisons (filled black circles along the x axis). A line linking multiple filled circles represents a column in which those differentially regulated genes are shared among more than one group comparison. For example, in the third column, there are 336 genes that are uniquely differentially regulated between both MEs versus MCs and FEs versus FCs, suggesting that these genes are responsive to endurance training independently of sex.

(B) PCA shows a strong clustering of MEs and FEs, whereas less separation is observed between the other three experimental groups. Principal-component 1 appears to separate the subjects based on sex, whereas principal-component 2 separates the subjects based on endurance training.

See also Table S2 for a complete list of all differentially regulated genes.

Short-Term Training Partially Reverses Metabolic Misregulation

In order to obtain a better understanding of how our transcriptomic data could inform the treatment of metabolic disease, we cross-referenced our lists of differentially regulated genes with two published studies examining gene expression in skeletal muscle samples before and after a training period in men with T2D (GEO: GSE19420) and women with metabolic syndrome (GEO: GSE43760). Correlations of absolute expression data between the current study's RNA-seq dataset and subjects before and after an exercise training program show that skeletal muscle gene expression in these trained T2D and metabolic syndrome subjects is most closely aligned with the highly trained endurance athletes (Figures 5A and 5C). Interestingly, many oppositely regulated genes were found between our endurance-trained groups (MEs versus MCs and FEs versus FCs) and the T2D and metabolic syndrome datasets (Figures 5B and 5D). At baseline, numerous oppositely regulated genes were present when comparing our data with skeletal muscle transcriptomic data from patients with T2D and metabolic syndrome (Figures 5B and 5D; Table S4). However, following an exercise-training program of either 1 year or 6 months, there is a decrease in the number of oppositely regulated genes when comparing our data to both studies (Figures 5B and 5D; Table S4). In total, exercise training for 1 year in men with T2D resulted in 31 out of 34 genes no longer being oppositely regulated between our dataset (MEs versus MCs) and the T2D dataset (Table S4). These "eliminated genes" that were enhanced in MEs versus MCs but inhibited with T2D at baseline are generally related to oxidative metabolism and mitochondrial structure (Table S4). The genes that were inhibited in MEs versus MCs but enhanced with T2D at baseline are related to the regulation of protein secretion and nuclear factor κB (NF-κB) signaling (Table S4). Similarly, 6 months of endurance training in women with metabolic syndrome resulted in 87 out of 105 genes no longer being oppositely regulated between our data (FEs versus FCs) and the metabolic syndrome dataset (Table S4). The genes that were originally enhanced in FE versus FC subjects and inhibited in metabolic syndrome at baseline were related to oxidative metabolism, protein metabolic processes, and vascular development (Table S4). The genes originally found to be inhibited in FEs versus FCs and enhanced with metabolic syndrome at baseline were associated with adipocyte differentiation, protein phosphorylation, and apoptosis (Table S4).

DISCUSSION

The present study offers extensive novel insights into the transcriptional differences that result over decades-long exercise training regimens in both women and men. Additionally, these data provide a solid picture of the sex differences in skeletal muscle gene expression that are present in untrained and endurance-trained individuals. Furthermore, in an analysis of existing gene expression data, we found that following short-term (6–12 months) exercise training programs, individuals with impaired metabolism shifted their gene expression to become more transcriptionally similar to our long-term endurance trained groups.

The physiological data presented in Figure 1 agree with several previous studies examining skeletal muscle adaptation to both endurance and resistance training. CS activity was found to be highest in the endurance-trained athletes, which is as expected based on previous work in the field (Holloszy and Booth,

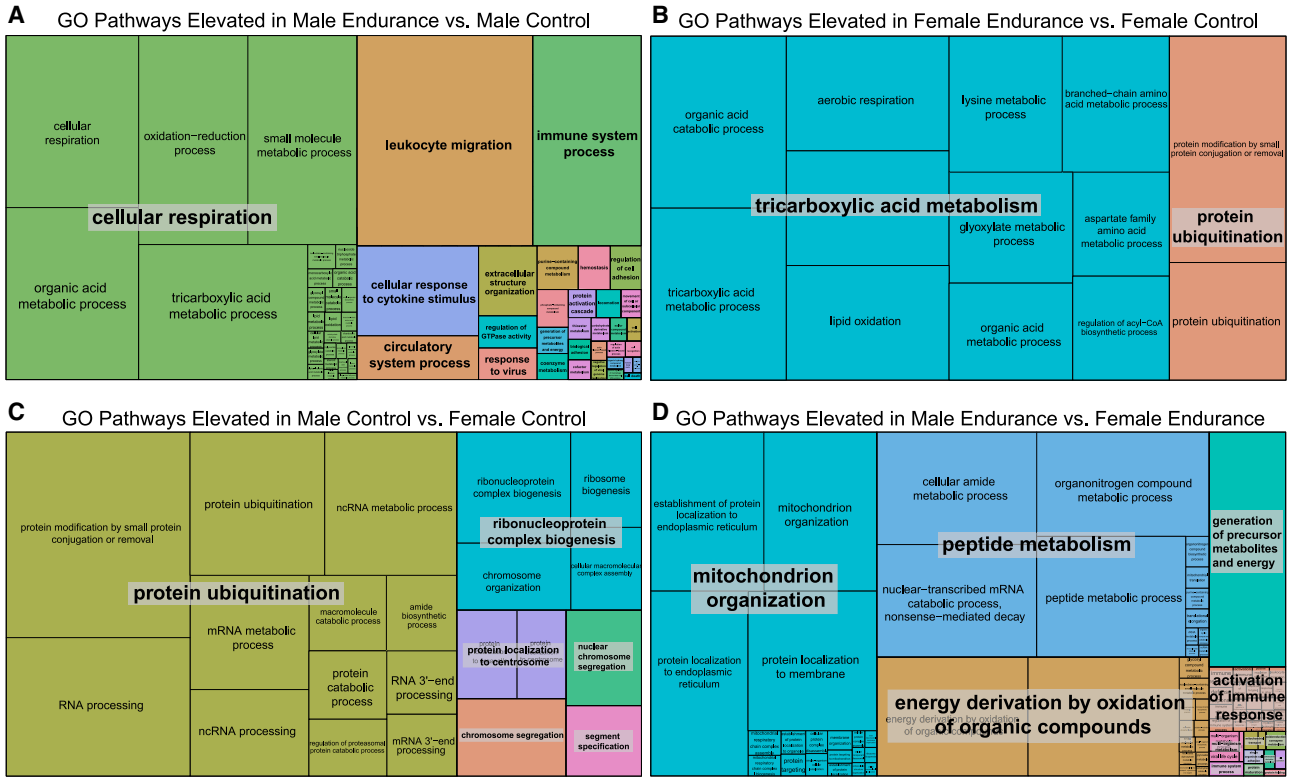

Figure 3. GO Pathway Analysis Examining the Effects of Long-Term Endurance Training and Sex

(A) REVIGO (reduce and visualize GO) TreeMap of GO terms elevated in response to long-term endurance training in men compared with controls.

(B) Elevated GO terms in response to long-term endurance training in women compared with controls.

(C) Significantly elevated pathways in MCs compared with FCs.

(D) Significantly elevated pathways in MEs compared with FEs. Pathways displayed all have a false discover rate (FDR) of <0.01.

See also Table S3 for a complete list of all significantly altered GO pathways and Figures S2–S4 for REVIGO TreeMaps of GO pathways inhibited with long-term endurance training (Figure S2), pathways elevated in FCs and FEs compared with MCs and MEs, respectively (Figure S3), and pathways elevated and inhibited in MSs compared with controls (Figure S4).

1976; Hoppeler et al., 1985). It is known that endurance activities increase the amount and activity of mitochondria in skeletal muscle. Muscle fiber area is indicative of overall hypertrophy in the muscle, which is traditionally associated with resistance training (Häkkinen and Keskinen, 1989; McCall et al., 1996). As expected, MSs had a greater muscle fiber cross-sectional area than controls. Although no significant difference was observed between MSs and MEs, this is not completely unexpected because it has been found that long-term endurance training, especially cycling, can increase myofiber area, although to a lesser extent than resistance training (Fry et al., 2014; Conceição et al., 2018). Indeed, more than 50% of our MEs and FEs were involved in cycling. This finding can also explain the greater cross-sectional area in the FE group than in the FC group. Finally, it is known that skeletal muscle fiber type can influence muscle performance (Gollnick et al., 1972; Costill et al., 1976; Fink et al., 1977; Saltin et al., 1977; Pette and Hofer, 1979). Our data show that endurance-trained athletes have a higher proportion of MyHC I, which is consistent with previous data showing that endurance-trained individuals have a propensity for a larger fraction of slow-twitch muscle fibers (Gollnick et al., 1972; Costill et al., 1976; Fink et al., 1977; Saltin et al., 1977; Pette and Hofer, 1979; Coggan et al., 1990). Furthermore, the prevalence of type IIa MyHC in strength-trained athletes is also consistent with the literature (Tesch et al., 1984; Adams et al., 1993). Finally, MyHC IIx are most prevalent in both FCs and MCs, which is in agreement with other studies that show type IIx muscle fibers are associated with low levels of physical activity (Larsson and Ansved, 1985; Tanner et al., 2002; Kosek et al., 2006; Fry et al., 2014). Although there is evidence of fiber-type shifts with physical activity, it should be noted that this is a cross-sectional study and we cannot infer whether the differences in fiber type are simply due to a genetic predisposition or if these differences arose from long-term training adaptations (Larsson and Ansved, 1985; Staron et al., 1990; Kosek et al., 2006; Fry et al., 2014).

Our transcriptomic data suggest that endurance training is a more powerful dictator of differential gene expression than either resistance training or sex in skeletal muscle following 72 h of no training. From the UpSet plot in Figure 2, it is clear that endurance training results in the highest amount of differential gene expression in both women (1,711 genes) and men (1,097 genes) compared with control groups. Similar to previous studies

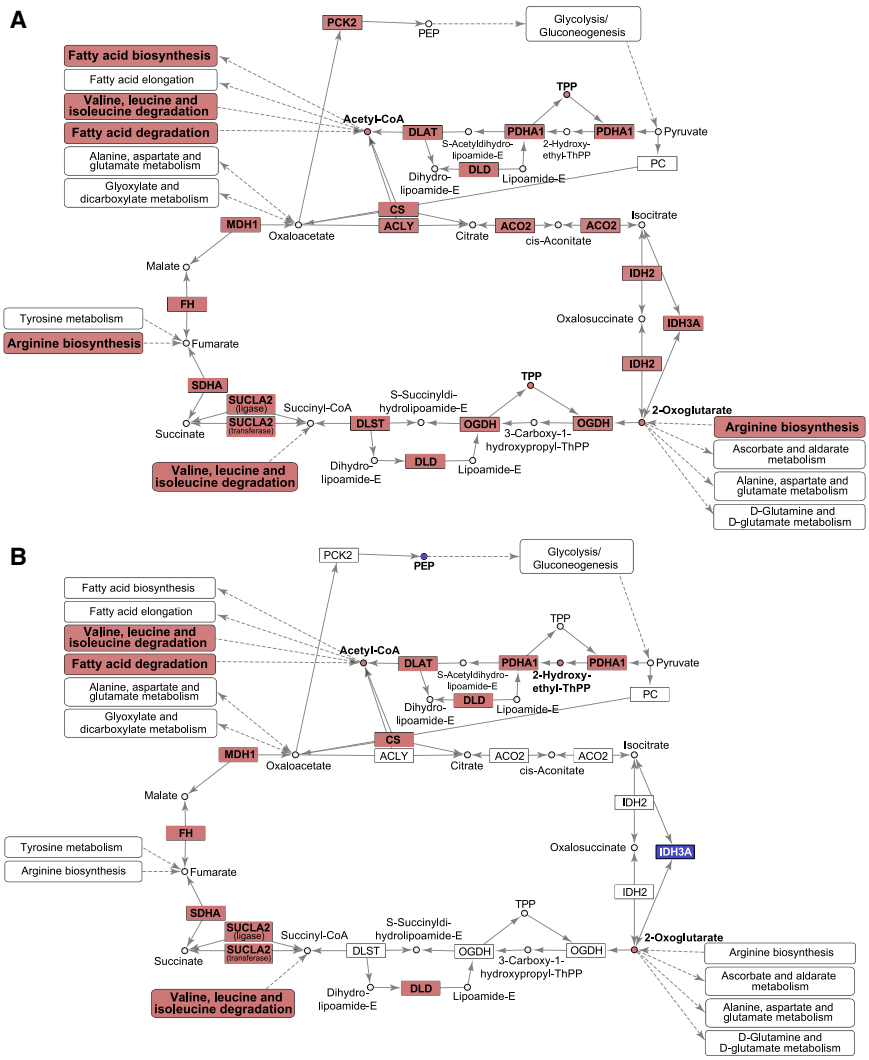

Figure 4. Genome-Scale Metabolic Modeling Reveals a Strong Alteration in Aerobic Metabolism in MEs and FEs

(A) Genome-scale metabolic model of the tricarboxylic acid (TCA) cycle in MEs shows that most of the TCA cycle and associated pathways are enhanced (salmon-colored) with endurance training compared with controls.

(B) Genome-scale metabolic model of the TCA cycle in FEs reveals enhanced expression, albeit to a lesser extent than with men (A), of many genes and pathways compared with controls. Rectangles represent genes, and circles represent metabolites. Gene expression levels are sourced from RNA sequencing data, and metabolite expression levels are predicted using metabolic modeling. Salmon-colored squares represent enhanced expression, white represents no change, and blue represents an inhibited expression compared with controls.

examining both the acute and chronic effects of endurance training on skeletal muscle, the current study shows a reprogramming of the skeletal muscle transcriptome to support aerobic respiration in both men and women (Pilegaard et al., 2000; Mahoney et al., 2005; Stepto et al., 2009; Lindholm et al., 2014a). Although this is not a surprising finding, the current study is the first to perform an analysis of the complete skeletal muscle transcriptome following decades-long training. Approximately 50% of genes that were found to be differentially regulated in MEs versus MCs were also found to be differentially regulated following a 3-month endurance-training protocol in males (Lindholm et al., 2014a). When comparing our data from FEs versus FCs to the same dataset, we found that approximately 30% of the same genes were differentially regulated (Lindholm et al., 2014a). This finding strongly suggests that some of the accumulated changes in the current study already exist following a 3-month training protocol. Overall, our data advance findings from previous exercise physiology studies and provide a wealth of new unbiased information into the gene expression differences that accumulate over a lifetime of regular exercise training.

This long-term accumulation of transcriptional changes likely plays a significant role in the health benefits that results from regular physical activity.

Previous work in a short term endurance training program of 3 months demonstrated a coordinated downregulation in the regulation of gene expression (Lindholm et al., 2014a). Furthermore, in a study examining long-term (≥8 years) adaptation to exercise training, it was also shown that pathways associated with transcription and translation were downregulated (Stepto et al., 2009). The current study also shows this to be the case in both MEs and FEs compared with controls (Figure S2). These data show a downregulation in pathways related to *inter alia*, RNA splicing, mRNA metabolic and catabolic processes, and translation initiation. Based on our study as well as previous studies, it is apparent that exercise training is associated with a downregulation in gene regulatory processes.

Many studies have found differences in fuel utilization in male versus female skeletal muscle, which has been observed at rest as well as following acute exercise interventions (Tarnopolsky et al., 1990; Welle et al., 2008; Lindholm et al., 2014b). In particular, it has been shown that skeletal muscle in men tends to favor peptide and carbohydrate oxidation, whereas skeletal muscle in women favors fatty acid oxidation. Our data reinforce these previous observations and demonstrate a strong enhancement in peptide metabolic processes in MCs, whereas FCs show elevated levels of lipid metabolism (Figure 3C; Table S3). Interestingly, these sex differences become less apparent with long-term endurance training, as can be observed in the decrease in peptide metabolism differences in ME versus FE subjects (Figure 3D) compared with controls (Figure 3C). Although peptide metabolism is still largely enhanced in MEs

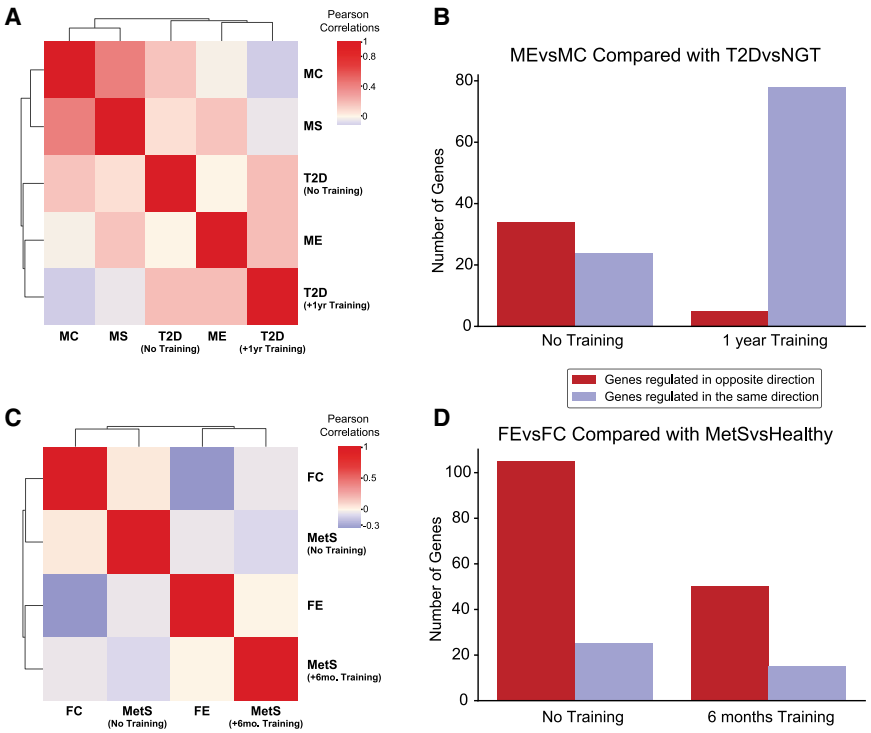

Figure 5. Endurance Athlete RNA Sequencing Data Compared with Two Published Studies Examining Gene Expression in Skeletal Muscle Samples before and after a Training Period in Men with Type 2 Diabetes (T2D) (GSE19420) and Women with Metabolic Syndrome (MetS) (GSE43760)

(A) Pearson correlations of absolute expression data between the current study's RNA sequencing dataset (MCs, MEs, and MSs) and T2D subjects before (T2D-no training) and after (T2D-+1yr training) a 1-year exercise training program. MEs and the exercise-trained T2D subjects cluster, demonstrating that skeletal muscle gene expression in these trained T2D subjects is most closely aligned with the highly trained MEs.

(B) A comparison of all genes found to be differentially regulated in the present study (MEs versus MCs) and T2D patients before and after a 1-year exercise program. These data show that the number of oppositely differentially regulated genes found between the current study and T2D subjects decreases following 1 year of exercise training by individuals with T2D.

(C) Pearson correlations of absolute expression data between the current study's RNA sequencing dataset (FCs and FEs) and women with MetS before (MetS-no Training) and after (MetS-+6mo. training) a 6-month endurance training program. FEs and the exercise-trained MetS subjects cluster, demonstrating that skeletal muscle gene expression in these trained MetS subjects is most closely aligned with the highly trained FEs.

(D) A comparison of all genes found to be differentially regulated in the present study (FEs versus FCs) and MetS patients before and after a 6-month exercise program. These data show that the number of oppositely differentially regulated genes found between the current study and subjects with MetS decreases following 1 year of exercise training by individuals with MetS.

See also Table S4 for a complete comparison of differentially regulated genes in the current study versus GEO: GSE19420 and GEO: GSE43760 and a list of all genes no longer oppositely regulated following a months-long training program.

versus FEs, it does not dominate the GO pathways like it does in the controls. This could be partly due to the fact that protein catabolism is increased with long-term endurance training in female athletes compared with controls (Figure 3B). Additionally, the pathways related to lipid metabolism that were enhanced in FCs compared with MCs were not found to be differentially regulated between FEs and MEs (Table S3). As such, it appears that, compared with controls, endurance training in men enhances the muscle's ability to oxidize lipids, whereas women seem to enhance their ability to use carbohydrates and peptides as fuel (Figure 3).

In line with the above observations, the data indicate that long-term endurance training lessens gene expression differences between men and women. With only 135 differentially regulated genes between MEs and FEs, the sexes appear to "converge" on a particular skeletal muscle transcriptome that may contribute to optimized skeletal muscle endurance. In fact, only 50 out of 452 of the differentially regulated genes found between MCs and FCs remained differentially regulated between the FEs and MEs. This finding indicates that most of the differentially expressed genes in untrained females versus males are altered with long-term endurance training. Although the transcriptomes of women and men converge with endurance training, there does appear to be an attenuation in terms of the extent to which women adapt to long-term endurance training. This is indicated in Figures 3D, 4A, and 4B, in which endurance trained men have significantly elevated pathways relating to oxidative metabolism. Figure 4A demonstrates that nearly all of the components in the TCA cycle are significantly enhanced in MEs compared with controls, whereas many of these genes are unchanged with long-term endurance training in women (Figure 4B). Furthermore, many of the pathways depicted in Figure 3D are related to mitochondria, despite our finding that CS activity was elevated in both MEs and FEs to similar extents. It should be noted that none of these pathways are found in the comparison of MCs versus FCs, suggesting that these differences do not exist in the untrained state. Instead, it appears that these changes arise following long-term endurance training, which supports the hypothesis that endurance gene expression in women is activated to a lesser extent than in men. This hypothesis could be supported by the attenuated inflammatory response that has been described in the skeletal muscle of recreationally active female subjects compared with men (Stupka et al., 2000; Tiidus, 2003). This has been hypothesized to be related to the intrinsically elevated estrogen levels in women, although the topic is unsettled in the literature (Hubal and Clarkson, 2009; Tiidus and Enns, 2009). The current study demonstrates that inflammatory pathways are indeed elevated in MEs

but not FEs compared with controls (Figure 3A; Table S3). Furthermore, MEs have a significantly enhanced expression in pathways relating to the activation of the immune response compared with FEs (Figure 3D). It is tempting to speculate that these differences in the inflammatory pathways could result in an attenuated adaptation to long-term training, but it is not entirely clear that these sex differences explain the differences between female and male athletes (Tiidus, 2003). Additionally, given the cross-sectional nature of this study, it must be noted that these differences could arise from disparate training intensities/frequencies and/or genotypic differences. Thus, our data do not necessarily support a muted response to endurance training in women compared with men, but previous studies do suggest that this is plausible.

Given the small number of genes that were detected to be differentially regulated with strength training (26 in total), it is difficult to make a concrete statement about how genes are regulated with long-term strength training. That said, many pathways were actually altered despite the low number of differentially regulated genes. Several of these pathways center around cellular energetics and mitochondria (Figure S4A; Table S3), which is in agreement with a previous cross-sectional study examining long-term resistance training in men (Stepto et al., 2009). However, we did expect to see more pathways related to resistance training, such as ribosomal biogenesis and protein translation, but these are most likely only transiently activated. That being said, the observed downregulation of pathways related to the negative regulation of cellular growth (Figure S4B) could provide some explanation for the larger hypertrophy observed in the MS group compared with controls. As there was a small number of genes that were differentially regulated with 15+ years of resistance training, it can be speculated that the genes involved in the adaptation to strength training are transiently expressed and do not remain elevated following 3 days of no training. Along these lines, previous studies have demonstrated that resistance training is a potent activator of gene expression acutely at both 1 and 4 h post-exercise (>400 differentially regulated genes) (Raue et al., 2012; Dickinson et al., 2018). However, it was shown that global differential gene expression at baseline following a 12-week progressive resistance training period is limited to only a few genes (Raue et al., 2012). As such, we hypothesize that many of the accumulated differences that accrue as a result of resistance training are at the protein level instead of the transcriptomic level. Thus, an extensive proteomic analysis is warranted, but this is beyond the scope of the present study.

Stepto et al. (2009) previously investigated how gene expression is altered with either long-term endurance or resistance training in men using a microarray analysis. Interestingly, the present study found 241 differentially regulated genes between endurance- and resistance-trained athletes compared with only 21 in the study by Stepto et al. (2009). They did find 263 genes to be upregulated with long-term training, but it is unclear which training background this is attributed to because their analyses were pooled into a single training group. The differences present between our study and Stepto et al. (2009) could originate from data generation methods (microarray versus RNA-seq), training histories, and/or subject age. Furthermore, as stated above, finding parallels between Stepto et al. (2009) and our study is further convoluted because endurance-trained and resistance-trained subjects were pooled for differential gene expression analysis. This pooling of the data prevents an accurate comparison between these two studies.

When comparing our endurance-trained groups to datasets from patients with T2D and metabolic syndrome, many genes were found to be differentially regulated in opposite directions at baseline (Figures 5B and 5D; Table S4). However, following a short-term exercise training program (6–12 months), the number of oppositely regulated genes decreased substantially. This suggests that even short-term exercise training programs are sufficient to alter the skeletal muscle transcriptome and bring it closer to the expression levels seen with long-term endurance training. This is further highlighted by the fact that, after 6+ months of exercise training, both the T2D and metabolic syndrome patients clustered closest with our ME and FE groups, respectively (Figures 5A and 5C). When looking at the function of the genes that were no longer oppositely regulated following several months of training in the patient cohorts, we find that the patients no longer show an upregulation of genes associated with pathways related to high blood glucose and insulin resistance (polyol and inositol metabolic processes and NF-κB signaling; Table S4; Chung et al., 2003; Lorenzi, 2007; Patel and Santani, 2009; Bevilacqua and Bizzarri, 2018). Additionally, following a 6- to 12-month exercise program, we observed that the patients with T2D and metabolic syndrome have a partially reversed misregulation of genes associated with healthy skeletal muscle metabolism (Table S4). This further indicates that months-long training programs have the potential to improve skeletal muscle metabolism and put patients on a path toward a healthy metabolic state. One caveat of this analysis is that it assumes that all of the changes in gene expression occurring with long-term training are beneficial and all of the changes in gene expression with T2D and metabolic syndrome have a negative impact on health.

The primary limitation associated with the current study is its inherent cross-sectional nature. This is a limitation because we cannot be certain that the differences we found are solely related to the training history of each subject. Instead, it is possible that some of the differences between the groups could be attributed to genetic variants instead of training history. That being said, the financial and experimental resources that would be required for a 15-year randomized longitudinal study are prohibitive. Furthermore, designing a control group that is prohibited from performing regular physical exercise for 15 years is ethically questionable. Given these issues with a randomized control study, the cross-sectional design present in the current study is warranted. Another limitation of this study is that most of our research subjects were of Caucasian decent. Thus, these results may not be representative for people of other racial backgrounds. Lastly, we were unable to recruit female strength-trained athletes, so we are unable to comment on how the skeletal muscle transcriptome in women is altered following 15 years of resistance training.

In summary, our data provide an extensive examination of the accumulated transcriptional changes that occur with decades-long endurance and resistance training in humans. Of note, we

observed that endurance training in both women and men drastically alters the transcriptome. These transcriptional changes with endurance training exceed the differences found between MCs and FCs as well as in strength-trained versus untrained men. Furthermore, these data provide evidence that skeletal muscle gene expression differences between men and women at baseline decrease following extensive endurance training. Additionally, only a few genes were differentially regulated between strength-trained athletes and controls. Thus, we hypothesize that the accumulated changes associated with resistance training are primarily relegated to protein levels instead of alterations in the resting baseline transcriptome. Finally, a comparative analysis revealed that following endurance-training programs of 6–12 months, individuals with impaired metabolism shifted their gene expression to become more transcriptionally similar to our long-term endurance-trained groups.

STAR★METHODS

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SUPPLEMENTAL INFORMATION

Supplemental Information can be found online at https://doi.org/10.1016/j.celrep.2020.107808.

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AUTHOR CONTRIBUTIONS

Conceptualization, M.A.C., M.E.L., A.M., and C.J.S.; Methodology, M.A.C., M.A., M.E.L., A.M., and C.J.S.; Software, M.A. and A.M.; Formal Analysis, M.A.C., M.A., and E.B.E.; Investigation, M.A.C., M.A., E.B.E., S.M.R., M.E.L., and C.J.S.; Writing – Original Draft, M.A.C. and M.A.; Writing – Review & Editing, M.A.C., M.A., E.B.E., S.M.R., M.E.L., A.M., and C.J.S.; Visualization, M.A.C. and M.A.; Supervision M.A.C., A.M., and C.J.S.; Project Administration, M.A.C., M.E.L., and C.J.S.; Funding Acquisition, M.A.C., A.M., and C.J.S.

DECLARATION OF INTERESTS

The authors declare no competing interests.

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STAR★METHODS

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Antibodies		
Anti-Laminin antibody produced in rabbit	Sigma-Aldrich	Cat# L9393, RRID:AB_477163
Goat Anti-Rabbit IgG (H+L) Antibody, Alexa Fluor 488 Conjugated	Molecular Probes	Cat# A-11008, RRID:AB_143165
Chemicals, Peptides, and Recombinant Proteins		
KH2PO4	Sigma-Aldrich	Cat# P9791
EDTA	Sigma-Aldrich	Cat# E7889
Triton X-100	VWR	Cat# 97063-864
Actyl-CoA	Sigma-Aldrich	Cat# A2056
Tris-HCL	Thermo Fisher scientific	Cat# 15568025
5,5′ Dithiobis (2-nitrobenzoic acid)	Sigma-Aldrich	Cat# D8130
Oxaloacetic acid	Sigma-Aldrich	Cat# O4126
HEPES	Sigma-Aldrich	Cat# H3375
Potassium fluoride	Sigma-Aldrich	Cat# 229814
Sodium orthovanadate	Sigma-Aldrich	Cat# S6508
Protease inhibitor	Roche	Cat# 11836153001
Acrylamide	Bio-Rad	Cat# 1610140
Bis-acrylamide	Sigma-Aldrich	Cat# 146072
Glycerol	Sigma-Aldrich	Cat# G5516
Glycine	Sigma-Aldrich	Cat# G8898
Ammonium persulfate	Sigma-Aldrich	Cat# A3678
TEMED	Sigma-Aldrich	Cat# T9281-25
Acetic acid	Sigma-Aldrich	Cat# A6283
Bovine serum albumin	VWR	Cat# 97061-422
Critical Commercial Assays		
Silver staining kit	Thermo Fisher Scientific	Cat# LC6100
Deposited Data		
RNaseq data	This manuscript	EGA: EGAS00001004367
Microarray data	van Tienen et al., 2012	GEO: GSE19420
Microarray data	Verheggen et al., 2016	GEO: GSE43760
Biological Samples		
Goat serum	Thermo Fisher Scientific	Cat# 31873, RRID:AB_2532167
Software and Algorithms		
Prism 7 for Mac	GraphPad Prism	RRID:SCR_002798
ZEISS ZEN Pro v2.0	ZEISS International	https://www.zeiss.com/microscopy/int/products/microscope-software/zen.html
ImageJ	NIH, https://imagej.net/Welcome	RRID:SCR_003070
Kallisto	Bray et al., 2016	RRID:SCR_016582
DEseq2	Love et al., 2014	RRID:SCR_015687
PIANO	Väremo et al., 2013a	RRID:SCR_003200
Jetset	Li et al., 2011	http://www.cbs.dtu.dk/biotools/jetset/
Limma	Ritchie et al., 2015	RRID:SCR_010943
Other		
Mounting medium	Vectashield	Cat# H-1000

RESOURCE AVAILABILITY

Lead Contact

Further information and requests for resources and reagents should be directed to and will be fulfilled by the Lead Contact, Mark Chapman (markchapman@sandiego.edu; mark.chapman@ki.se).

Materials Availability

This study did not generate new unique reagents.

Data and Code Availability

The RNA-sequencing data have been deposited at the European Genome-phenome Archive (EGA) which is hosted at the EBI and the CRG, under accession number EGA: EGAS00001004367. The data of individuals with diseases can be found at GSE19420 and GSE43760. The code used for processing our data can be found at https://github.com/sysmedicine/ChapmanEtAl_2020_1.

EXPERIMENTAL MODEL AND SUBJECT DETAILS

Ethical approval

This study was approved by the Regional Ethical Review Board in Stockholm, Sweden (application 2016/590-31) and conformed to the Declaration of Helsinki. Prior to performing the experiment, each subject was provided a detailed description of all procedures as well as potential complications. Written and verbal consent were both attained, and the subjects were informed that they may withdraw consent at any time during the experiment.

Human subjects

Forty healthy human volunteers aged 34-53 were recruited as subjects for this study (Table 1). These research subjects came from one of the following groups: endurance-trained male (ME) and female athletes (FE) with over 15 years of training, strength-trained men (MS) with over 15 years of training, and untrained (physical activity $\leq$ 2/week) age-matched male (MC) and female subjects (FC) as controls. We were unable to recruit women with over 15 years of resistance, and no endurance, training history.

METHOD DETAILS

Subject screening

Prior to coming to the laboratory, the potential research volunteers filled out a health and physical activity questionnaire to determine if they met the inclusion criteria. Untrained subjects with a BMI > 26 were excluded to minimize the influence of excess adipose tissue. Subjects with a history of any of the following were excluded: chronic disease, steroid and/or other performance enhancing drug use, smoking, or fainting during physical exercise. In order to pass this prescreening process, individuals had to belong to one of the following groups: involved in vigorous endurance training (running and cycling) over three days per week on average during the past 15 years, participation in heavy resistance training (powerlifting and Olympic lifting) three or more days per week on average over the past 15 years, or have had limited to no physical activity over the past 15 years. Additionally, since the goal of this study was to separate the effects of endurance and strength training, individuals with a training history in both endurance and strength training were excluded (i.e., individuals involved in CrossFit).

Performance testing

Following prescreening, 50 individuals that fit our inclusion criteria were brought into the lab for performance tests. These tests were used to quantify the fitness level and strength of the potential research subjects. To assess muscle strength, an isokinetic knee extension test at 90 deg/s was performed using the Biodex Isokinetic System (System 4 pro, Biodex medical systems, New York City, NY, USA) to measure maximal quadriceps torque production in each leg. Prior to testing, each subject was familiarized with the machine and the testing protocol. During the test, the subjects performed three maximal knee extensions, and the highest value from each leg was recorded. To assess endurance capacity, a maximal oxygen uptake (VO_2 peak) value was measured on a bicycle ergometer by incrementally increasing the resistance until exhaustion. Respiratory gases were measured throughout the VO_2 test (Sensor Medics Vmax 229; Intra Medic AB, Bålsta, Sweden). A plateau in oxygen consumption and a respiratory exchange ratio greater than 1.1 were achieved during the VO_2 test. Heart rate and perceived exertion (Borg Scale 6-20; Borg, 1970) were recorded every minute and every second minute, respectively. A Borg Scale value greater than 17 was achieved during all tests. An endurance-trained athlete was included into the endurance group if his/her VO_2 peak was above the 90th percentile for their age as determined by the LIV 2000 Study from the Swedish School of Sport and Health Sciences (Ekblom-bak et al., 2011). Additionally, the subject's peak knee extension torque had to be at least 1 standard deviation below the mean/average peak torque in resistance-trained group. A resistance-trained athlete was included if his peak knee extension torque was at least 2 standard deviations above the age-matched control group, and his VO_2 peak was below the 90th percentile for his age group. A subject was included into the control group if his/her VO_2 peak was below the 75th percentile for their age group, and their peak knee extension torque was at least 2 standard deviations

below the resistance-trained group. Of the 50 individuals that we screened, 40 were included into the study (7 male controls, 8 female controls, 9 endurance-trained males, 9 endurance-trained females, and 7 strength-trained males).

Biopsy collection

Included subjects were brought back to the laboratory at least two weeks after the performance testing session. Prior to the skeletal muscle biopsy collection subjects were instructed to refrain from: physical activities for 72 hours, drinking alcohol for 48 hours, taking medications containing acetylsalicylic acid for 1 week, and consuming beverages with caffeine the morning of the biopsy. All biopsy collections took place at the same time during the morning. Subjects were instructed to eat a standardized breakfast 3 hours before their scheduled biopsy collection. The biopsy site was disinfected with chlorhexidine. For local anesthesia, approximately 50 mg of Carbocaine (10mg/ml) was injected into the skin overlaying the vastus lateralis of both legs. Loss of sensation was confirmed before moving forward with biopsy collection. Small incisions in the skin and fascia were made with a scalpel over the distal vastus lateralis. Muscle biopsies were then taken with a 5 mm Bergström biopsy needle with suction (Bergström, 1962). Muscle samples were immediately frozen in liquid nitrogen-cooled isopentane and stored at −80°C.

Citrate synthase activity

10 mg of skeletal muscle sample was homogenized in 0.5 mL buffer (50mM KH_2PO_4, 1mM EDTA and 0.05% Triton X-100) in a bead homogenizer. Samples were then rotated for 15 minutes at 4°C and centrifuged at 1400*x* g for 1 minute. The supernatant was collected and protein content was measured with the Bradford assay. Samples were stored at −80°C until further processing. To measure citrate synthase activity, 2 μL of muscle homogenate was loaded in triplicate in a 96-well clear-bottomed plate. 188 μL of reaction buffer master mix (100 mM Tris-HCl [pH 7.5], 30 mM Actyl-CoA and 10 mM 5,5′-Dithiobis-[2-nitrobenzoic acid]) was added to each well. An oxaloacetic acid (OAA) solution (10μl/well: 10mM oxaloacetic acid in Tris-HCl) was added immediately before the plate was loaded into a spectrophotometer. 2 μL of reaction buffer master mix was added to three wells as a negative control. Absorbance at a wavelength of 412 nm was measured every minute for 15 minutes. Citrate synthase activity in each sample was determined as previously described (Bass et al., 1969) and the calculated value was standardized by the amount of protein loaded (determined by the Bradford assay).

Myosin heavy chain

30 mg of skeletal muscle sample was homogenized in 0.6 mL buffer (20 mM HEPES, 150 mM NaCl, 5 mM EDTA, 25 mM KF, 1 mM Na_3VO_4, 20% (v/v) glycerol, 0.5% Triton x-100 and protease inhibitor (Roche, Basel, Switzerland)) in a bead homogenizer. Samples were then rotated for 45 minutes at 4°C. Protein concentration was determined with the Bradford assay, and samples were diluted to a concentration of 20 ng/μl then stored at −80°C.

The proportion of each myosin heavy chain isoform (I, IIa, IIx) was then determined by gel electrophoresis as previously described (Mizunoya et al., 2008). In summary, samples were run on a 1.5 mm thick 8.5% polyacrylamide (Acrylamide/bis-acrylamide ratio of 99:1) separating gel (35% v/v glycerol, 200mM Tris [pH 8.8], 100mM glycine, 0.35% w/v SDS, 0.1% w/v ammonium persulfate, 0.05% v/v TEMED) and a 4% polyacrylamide (acrylamide/bis-acrylamide ratio of 99:1) stacking gel (10% v/v glycerol, 70mM Tris [pH 6.8], 4mM EDTA, 0.4% w/v SDS, 0.1% w/v ammonium persulfate, 0.05% v/v TEMED). Once the gels were polymerized, 10 μL of muscle homogenate was mixed with high-glycerol loading buffer (10 μl) for a final concentration of 10 ng/μl and heated to 99°C for 5 minutes. Samples were vortexed then loaded warm in the gel (5 μl/well). Gel electrophoresis was run for 22 hours at 4°C, where the current was limited to 40 mA for the first 40 minutes. Following gel electrophoresis, a commercial silver staining kit (Cat. No. LC6100, Thermo Fisher Scientific, St Louis, Missouri) was used to visualize the bands. The manufacturer's instructions were followed directly for a 1.5 mm thick gel with reduced samples: the gel was fixed for 20 minutes (ultrapure water, methanol, acetic acid) followed by two 60-minute incubations in a gel sensitizing solution (ultrapure water, methanol, gel sensitizer). Subsequently, the gel was incubated for 30 minutes in a staining solution before it was washed twice in ultrapure water for ten minutes. The gel was incubated in a developing solution for 3-15 minutes. When a desired staining intensity was reached, a stopping solution was added and the gels were incubated for 20 minutes. Finally, the gel was washed 3 × 20 minutes in ultrapure water. After staining, gels were imaged and quantified by densitometry using ImageJ software (National Institutes of Health, Bethesda, MD) to determine the relative MyHC isoform distribution.

Myofiber area

Myofiber cross sectional area was determined from histological sections as previously described (Minamoto et al., 2007; Meyer and Lieber, 2012; Chapman et al., 2014). Briefly, 10 μm transverse sections were cut using a Leica CM3000 Cryostat (Germany) at −20°C. The sections were mounted on microscope slides (Cat. No. J1800AMNT, Thermo Fisher Scientific, St Louis, Missouri), air-dried at room temperature, and stored at −80°C. In order to determine myofibrillar cross sectional area, tissue sections were stained with an antibody against laminin (1:400; Cat# L9393, RRID:AB_477163; Sigma-Aldrich, St. Louis, Missouri). A standard immunofluorescence protocol was followed: sections were encircled with a liquid blocker pap pen (Dako-pen, Agilent Technologies) and washed twice in PBS for 5 minutes. Samples were first blocked for 30 minutes with 1% BSA, followed by 15 minutes of 15 μl/ml normal goat serum in 1% BSA. Samples were incubated overnight with a rabbit polyclonal antibody against laminin in 1% BSA at 4°C. The following day, sections were washed (3 × 5 minutes) in PBS and incubated with an Alexa Fluor 488 goat anti-rabbit secondary antibody (1:500; Cat#

A-11008, RRID:AB_143165; Molecular Probes, Eugene, Oregon) in 1% PBS with 15 μl/ml normal goat serum for 1 hour at room temperature in the dark. Sections were washed and a coverslip was mounted using Vectashield mounting medium (Cat. No. H-1000; Vector Laboratories, Burlingame, CA, USA).

Stained sections were imaged using a 10x objective (Zeiss Axiocam 503 mono (AxioImager, Zeiss, Oberkochen, Germany). Muscle fiber cross sectional area was analyzed on laminin-stained sections using an ImageJ (ImageJ, RRID:SCR_003070) macro as previously described (Meyer and Lieber, 2012). Muscle fibers were only quantified within a range of 991-20661 μm^2 to eliminate vascular structures and optically fused fibers. Furthermore, circularity of analyzed muscle fibers was limited to between 0.5-1 to avoid analyzing obliquely cut fibers.

RNA isolation and quality control

30 mg of muscle sample was homogenized in 1.0 mL TRIzol reagent (ThermoFisher Scientific, Waltham, MA, USA) using a bead homogenizer. RNA was isolated from all samples using the standard TRIzol method as outline in the manufacture's protocol. To maximize RNA yield, 10 μg of RNase-free glycogen (R0551, ThermoFisher Scientific, Waltham, MA, USA) was added along with 0.5 mL isopropanol during the precipitation step and RNA was allowed to precipitate overnight at −20°C. 5 μg of RNA from each subject was treated with DNase (DNA-free kit, ThermoFisher Scientific, Waltham, MA, USA). The manufacturer's instructions were followed and the final RNA concentration was measured with absorbance at 260 nm. Prior to performing RNA sequencing, the resulting DNA-free RNA samples were analyzed for quality on a 2100 Bioanalyzer according to the manufacturer's protocol (Agilent Technologies, Santa Clara, CA, USA). RNA integrity numbers (RIN) were calculated automatically by the 2100 expert software.

RNA sequencing

RNA library preparation and sequencing was performed at the National Genomics Infrastructure – Sweden. Strand-specific TruSeq RNA libraries using poly-A selection were prepared. All 40 samples were then multiplexed in 1 lane and sequenced (2x50bp paired end) on the Illumina NovaSeq 6000.

QUANTIFICATION AND STATISTICAL ANALYSIS

Statistical significance was set to $p < 0.05$ and data are presented as standard error of the mean (SEM) unless otherwise stated. All individuals (n = 40: 17 and 23 female and male individuals, respectively) were included in the statistical analyses.

Subject characteristics and Performance data

Determining differences in subject characteristics and physical performance outcomes between study groups were performed via one-way ANOVAs and t tests, calculated in the software package Prism, for male and female subjects respectively.

Biochemical analyses

For CS-activity and myofiber area, one-way ANOVAs and t tests, for male and female subjects respectively, were performed to determine differences between groups. Additionally, for comparisons of the distribution of MyHC isoforms between study groups, two-way ANOVAs were performed for both male and female subjects. Analyses were calculated in the software package Prism.

Sequencing data analysis

Raw counts and Transcripts per million (TPM) value of each sample was generated from the RNA-seq data using Kallisto (Bray et al., 2016). Raw counts were subsequently used as the input to DESeq2 (Love et al., 2014) for identifying differentially expressed genes (DEG). We then used the fold changes and p values from the DEG to perform functional analysis, specifically enrichment analysis with GO Biological Process and KEGG Pathways. The functional analysis was done via R package called PIANO (Väremo et al., 2013a), with gene-set collection retrieved from MSigDB (Subramanian et al., 2005). For both analyses, we filtered the result with FDR of 5%. REVIGO was used to summarize and visualize Gene Ontology terms (Supek et al., 2011).

Sequencing data graphically compared with the creation of an UpSet plot (Lex et al., 2014). An interactive version of this plot can be accessed by visiting: http://vcg.github.io/upset/, clicking 'load data' in the menu bar, entering the JSON path link below and clicking 'submit': https://raw.githubusercontent.com/markchapman10/RNAseq/master/UpsetR_input_github.json

Furthermore, we integrated the RNA-seq data with Genome-Scale Metabolic Models (GEM), to identify the metabolites that are affected by the transcriptional changes. Generic human model, HMR version 2.00 (Mardinoglu et al., 2014), were used to generate the genes-metabolites network. Subsequently, we performed reporter metabolites analysis by using PIANO.

Metabolic syndrome sequencing datasets

RNA sequencing data from the current study was compared with two existing microarray datasets that examined the influence of exercise training programs on subjects with type II diabetes (GSE19420; van Tienen et al., 2012) and with metabolic syndrome (GSE43760) (Verheggen et al., 2016). van Tienen et al. (2012) subjected T2D patients to a 1-year supervised exercise program involving primarily endurance training (high-intensity interval training of 4-8 cycling bouts of 30/60 s at 50%–60% maximum power and 30-minute cycling at an average intensity of ~80% maximum heartrate) and some resistance training (progressive

resistance-type exercise training targeting 5-7 major muscle groups of the arms and legs with 2 × 12 repetitions, at ~65% of 1 repetition maximum strength) for an average of two sessions per week. The study performed by Verheggen et al. (2016) involved a 6-month supervised endurance exercise program consisting of 30-minutes cycling three times per week 65% of the individual's heart rate reserve.

Microarray probes were converted into genes and selected with Jetset package in R (Li et al., 2011). After selecting the probe, differential gene expression (DGE) analysis of the data was done with the limma package in R (Ritchie et al., 2015). Subsequently, the DGE results were used to perform functional analysis with PIANO (Väremo et al., 2013a).

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Supplemental Information

Skeletal Muscle Transcriptomic Comparison between Long-Term Trained and Untrained Men and Women

Mark A. Chapman, Muhammad Arif, Eric B. Emanuelsson, Stefan M. Reitzner, Maléne E. Lindholm, Adil Mardinoglu, and Carl Johan Sundberg

Table S1: Related to Table 1. Self-reported training history of all exercise-trained subjects over the past 15+ years.

Training Duration (years)	Average training frequency (days/week)	Average training frequency (hours/week)	Primary Activities
Male Endurance			
20+	5-6	3-6	Running/cycling
15-17	5-6	6-9	Running/cycling
20+	3-4	3-6	Running/cycling/skiing
20+	3-4	1-3	Running
20+	5-6	6-9	Cycling, occasional running
20+	4-5	3-9	Running/cycling
20+	5-6	6-9	Running/cycling
15-17	4-5	6-9	Running/cycling
20+	7	>10	Running/cycling/skiing
Female Endurace			
20+	5-6	>10	Running/cycling
20+	5-6	6-9	Running/cycling
20+	5-6	6-9	Running/cycling
20+	5-6	6-9	Running/skiing
15-17	5-6	6-9	Running
20+	3-4	3-6	Running
18-20	3-4	3-6	Cycling
20+	5-6	6-9	Cycling
18-20	5-6	6-9	Running
Male Strength			
15-17	3-4	1-3	Full body strength training
20+	3-4	3-6	Full body strength training
20+	5-6	6-9	Competetive powerlifting
20+	3-4	6-9	Full body strength training
20+	4-5	3-6	Full body strength training
20+	5-6	6-9/>10	Full body strength training
20+	3-4	3-6	Competetive powerlifting

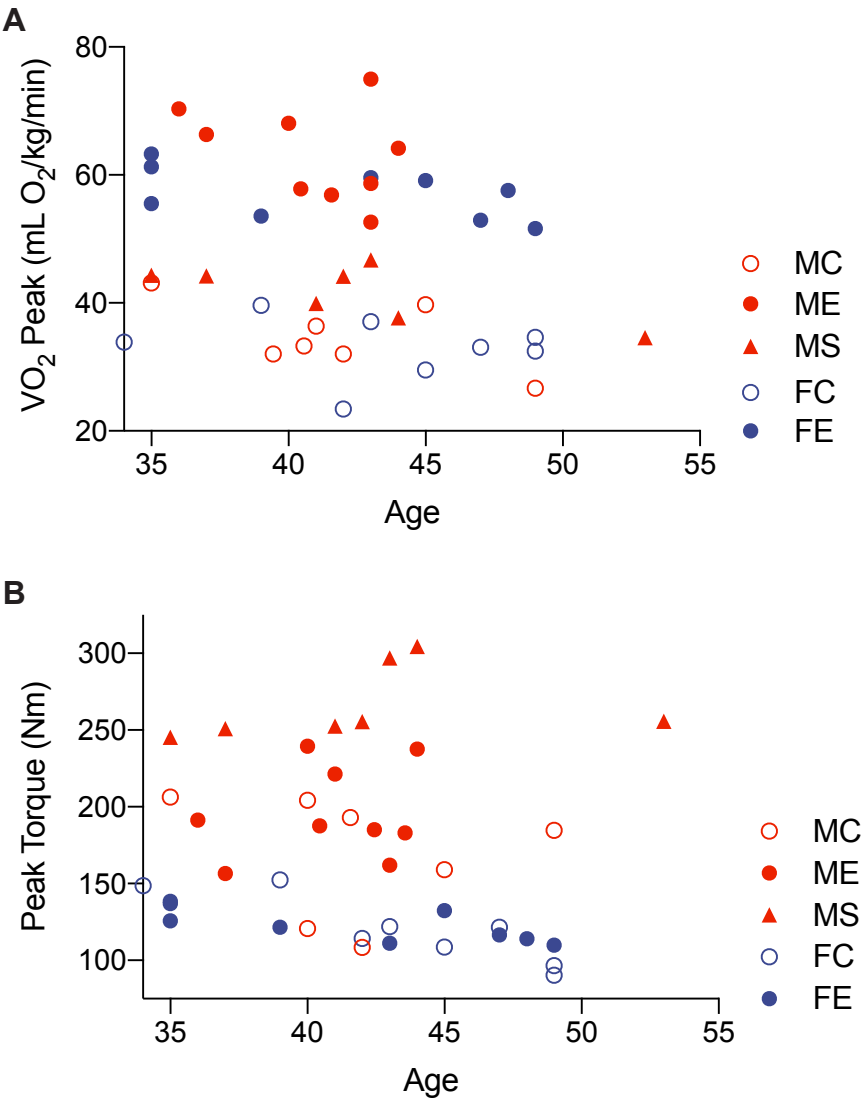
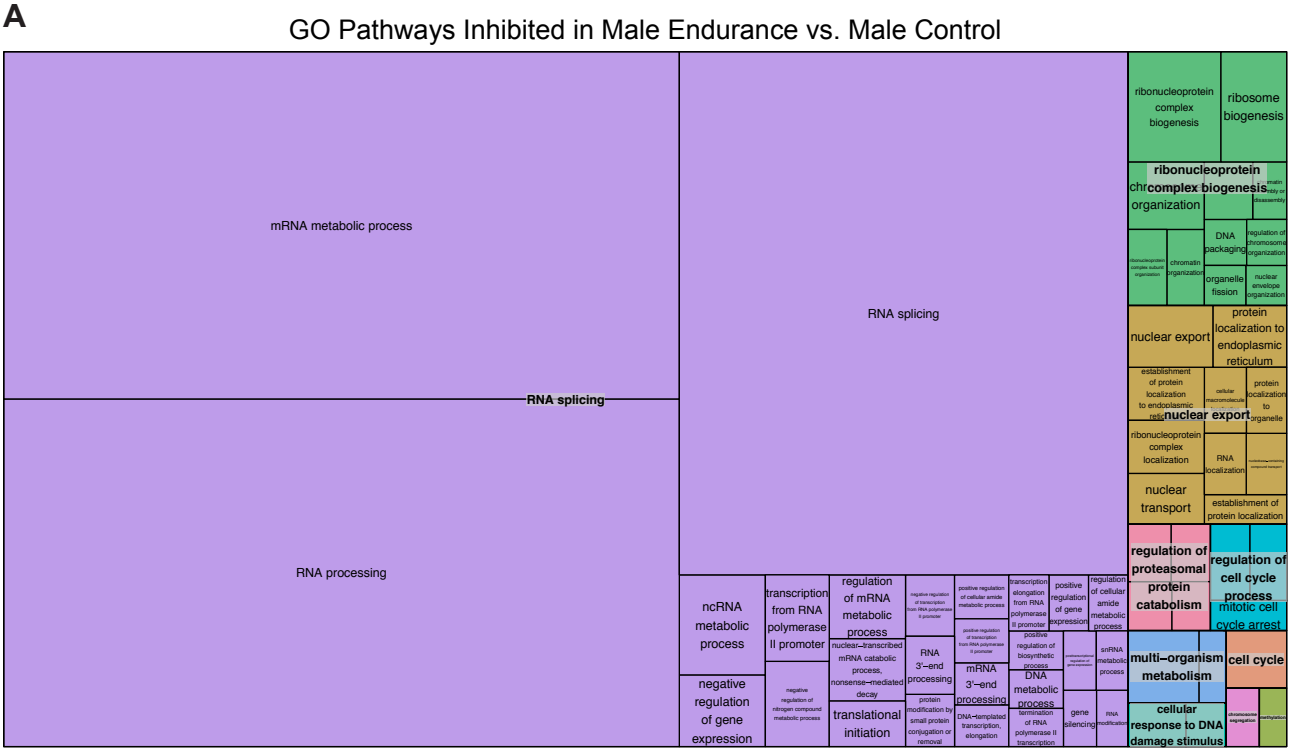


Figure S1: Related to main text Figure 1A and 1B. A clear separation in both VO2 peak (**A**) and peak leg torque (**B**) was present between the experimental groups. Of note, in the VO2 peak data there is no overlap between the endurance-trained athletes and the other groups. Additionally, no overlap in peak leg torque was present between the resistance trained athletes and the other groups. These data demonstrate the clear physiological differences present in our subjects.

A



B

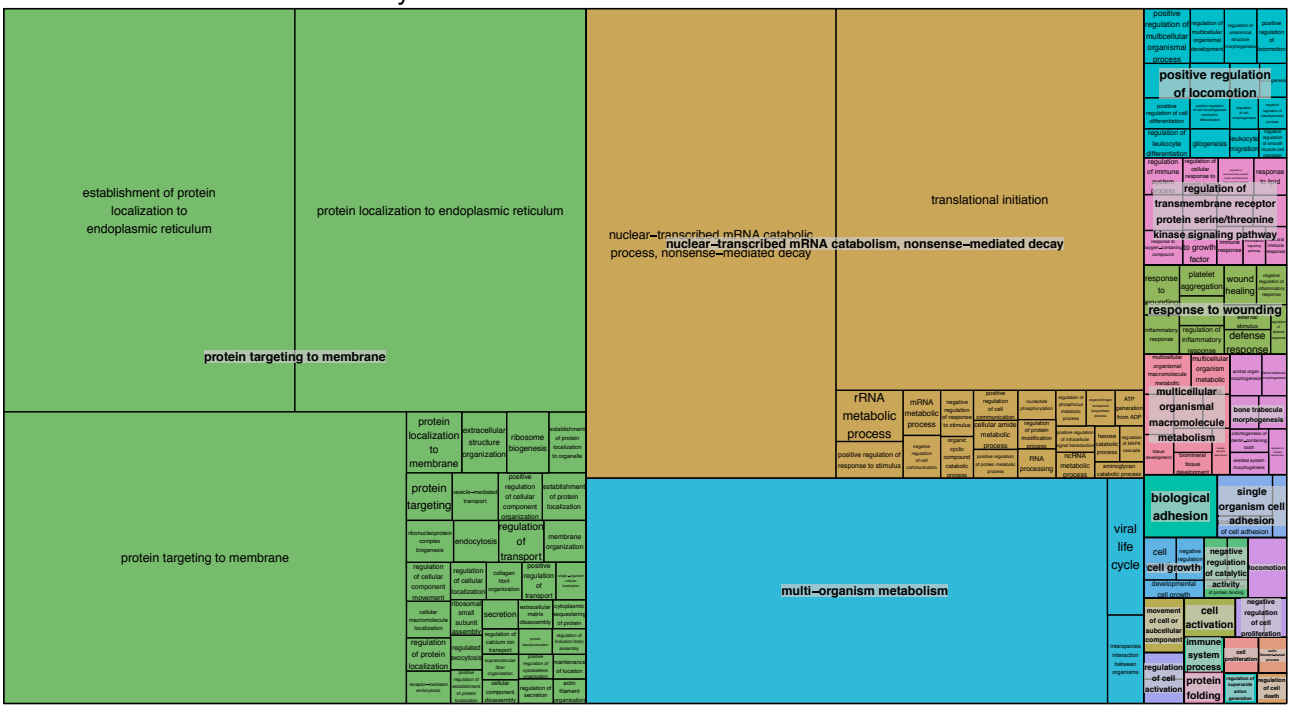


Figure S2: Related to main text Figure 3. REVIGO TreeMap of inhibited GO terms in response to long-term endurance training in males (**A**) and females (**B**) compared with controls. Pathways displayed all have FDR<0.01.

A

GO Pathways Elevated in Female Control vs. Male Control

B

GO Pathways Elevated in Female Endurance vs. Male Endurance

Figure S3: Related to main text Figure 3. REVIGO TreeMap of elevated GO terms in female controls compared with males (**A**) and in female endurance athletes compared with male endurance athletes (**B**). Pathways displayed all have FDR<0.01.

Figure S4: Related to main text Figure 3. REVIGO TreeMap of elevated (**A**) and inhibited (**B**) GO terms in male strength-trained athletes compared with controls. Pathways displayed all have FDR<0.01.